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PRINCIPAL INVESTIGATOR: Darryl Shibata, M.D.

CONTRACTING ORGANIZATION: University of Southern California
Los Angeles, California 90033

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Darryl Shibata, M.D.

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University of Southern California
Los Angeles, California 90033

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A precise understanding of the genetic changes which herald incipient transformation would be invaluable for accurate early diagnosis and a better understanding of breast cancer pathogenesis. Towards this goal, we tried to identify clonal breast populations based on the detection of only one X-chromosome androgen receptor allele after differential methylation sensitive restriction enzyme digestion. Although this analysis was suitable for large amounts of DNA, it was unreliable when small cell numbers were analyzed. A novel strategy to dilute the DNA molecules to essentially single alleles after restriction digest prior to PCR was able to identify small clonal and polyclonal populations but was extremely time consuming. Further development of this strategy may improve the ability to reliably identify small clonal cell populations in the breast.

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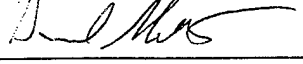

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4) TABLE OF CONTENTS

TITLE: GENETIC EVIDENCE OF EARLY BREAST CANCER

1) INTRODUCTION:

Cancer is thought to arise from a series of mutations which culminate in malignancy (1,2). The exact number, timing, and types of these mutations are unclear in breast cancer. Although the earliest genetic alterations in breast cancer are unknown, the earliest neoplastic lesions should be clonal. The goal of this proposal was to identify clonal regions of breast epithelium in order to further understand how early neoplasia arises. To accomplish this goal, we analyzed X-chromosome inactivation of the androgen receptor gene, since neoplastic regions should exhibit inactivation of the same X-chromosome whereas non-neoplastic regions should exhibit random inactivation of both X-chromosomes.

Numerous studies have demonstrated that breast cancer is clonal based on X-chromosome inactivation (for example 3,4). Additional studies have been published since the submission of this proposal which clearly demonstrate that breast cancers are clonal (5-7). Therefore, we saw little need to further replicate these findings. In addition, two other studies have indicated that both normal (8) and hyperplastic or ductal carcinoma in situ (DCIS) (9) are clonal by X-chromosome inactivation analysis. Of note, apparently clonal mutations in p53 and various chromosomal deletions have been reported for DCIS (10). Because of the important implications of small clonal populations in the pathogenesis of breast cancer, we sought to confirm these findings and improve the methodology of very small tissue clonal regional analysis.

This topographic analysis requires the ability to microdissect at high resolution the thin layers of epithelium from surrounding stroma and tumor cells. The method for microdissection was selective ultraviolet radiation fractionation (SURF) (11). Small numbers (50-200) of cells (a single duct or lobule) with specific phenotypes (normal, premalignant, and tumor) (12) on a stained tissue section were microscopically identified and covered with very small ink dots. UV radiation then destroys everything except the DNA in the desired protected cells, and subsequent PCR should reveal their specific genotypes. X-chromosome inactivation provides the earliest evidence of clonal proliferation and can be used to identify clonal populations even if their underlying mutations are unknown (13,14). The topographical distributions of X-chromosome inactivation in the primary tumor, and its extension into adjacent non-neoplastic epithelium, can define the presence and extent of the altered epithelium which precedes transformation.

6) BODY: Progress will be discussed in reference to the tasks identified in the proposal's statement of work:

STATEMENT OF WORK: GENETIC PROFILE OF EARLY BREAST CANCER

TASK 1: Optimize X-chromosome inactivation assay for SURF (Months 1-3)

A) Obtain female cell lines (N=4)
B) Make various mixtures of formalin fixed, paraffin embedded cell lines
C) Compare results from SURF with DNA purified from cell lines

TASK 2: Determine the topographic distribution of X-chromosome inactivation in normal, premalignant, and malignant breast epithelium (Months 3-24)

A) Obtain fixed breast cancer specimens (40 per year)
B) Optimize X-chromosome analysis for PCR and SURF
C) Determine clonal patterns of X-chromosome inactivation in tumor tissue
D) Determine if the same clonal inactivation patterns extend into adjacent preneoplastic and normal epithelium
E) Analyze normal breast tissues

Task 1: We obtained and isolated DNA from seven breast cancer cell lines (MCF-7, ER75, HEL1-8, BT474, MDAMB-453, MRF-7, MDA-BB) and have made artificial mixtures of known clonal compositions for analysis in Task 2.

Task 2 A: We have obtained formalin fixed tissue blocks from 40 breast cancer patients. They have been examined, and appropriate areas of tumor and adjacent normal tissue have been identified. The DNA has been extracted in bulk from the 40 breast cancers (both normal DNA and tumor DNA from the same patient).

Task 2 B: We have synthesized eight different PCR primers sets which span the methylation sensitive restriction enzyme sites (HpaII and HhaI) immediately 5′ to the triplets CAG repeats androgen receptor located on the X-chromosome. The primary method needed for this study is the ability to distinguish between polymorphic methylated androgen receptor loci. Restriction digestion using methylation sensitive enzymes (HpaII, HhaI) will cut only the unmethylated allele. Subsequent PCR with primers located outside of the restriction sites should only amplify the methylated (ie uncut) allele. Therefore, clonal populations are identified by the amplification of only a single allele whereas polyclonal (reactive) populations would yield both alleles (15-17).

Using various sets of these PCR primers, we have identified 10 out of the 40 breast cancers from Task 1 which are well suited for further analysis since they are polymorphic for the number of CAG repeats, with their two different alleles easily distinguished on small acrylamide minigels. We have been able to demonstrate clonal X-chromosome inactivation using restriction enzyme digestion and PCR in these breast cancers, using bulk extracted DNA.

A major problem has been encountered when the assay is scaled down to analyze the small amounts of DNA present in microdissected regions. With small numbers of cells (less than 1,000), the assay becomes unreliable with polyclonal populations demonstrating clonal patterns and clonal populations demonstrating polyclonal

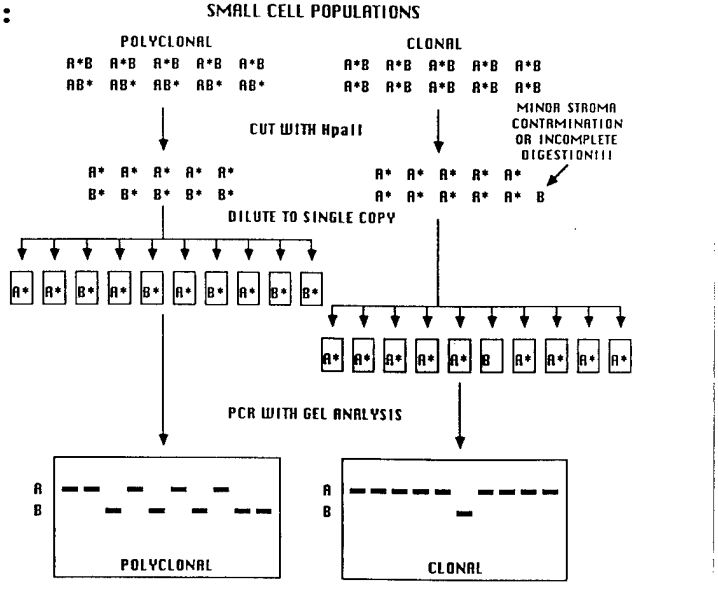
patterns. This lack of reliability with small numbers of cells is a major problem since the primary goal of this proposal is the detection of small clonal populations.

The primary problem with the current approach appears to be the inability of PCR to accurately represent the proportion of alleles present in the original sample. Because small numbers of cells must be analyzed, a large number of PCR cycles (40-46) are necessary to achieve the necessary sensitivity. Unfortunately, if a minor residual fraction of one androgen receptor allele is present (still indicating a clonal population) the large number of PCR cycles obscures the true starting fraction, and both alleles are amplified to similar extents, leading to "false" negatives.

The contamination of clonal populations by the "unmethylated" allele is due to two sources. First, all breast cancers are contaminated by normal stroma cells. Even with the best microdissection, approximately 5-10% contamination by stroma cells is inevitable. Second, restriction digestion may be incomplete leading to a small fraction of uncut but unmethylated alleles. Therefore, clonal breast epithelial populations will always harbor a small fraction of contaminating "nonclonal" alleles.

Another problem is with polyclonal populations. Because formalin fixed, paraffin embedded tissues are used, DNA degradation is present. The extent of this degradation cannot be predicted and sometimes only a small number of molecules can be amplified. However, if only a small number of DNA molecules are suitable for PCR amplification, then the same allele may be selected by chance from even polyclonal populations. Therefore, using known reactive and monoclonal (ie tumor) tissues, reliable detection has not been possible when small numbers of cells are analyzed.

To overcome these informative failures, we have changed the strategy. Although PCR can amplify small numbers of alleles to detectable levels, the primary problem is the inability of PCR to accurately represent the proportion of alleles present in the original sample. Therefore, we have altered the assay such that PCR is no longer used to distinguish between different allelic proportions. Instead, separation of the alleles occurs prior to PCR and PCR is only used to detect the alleles. This is illustrated below:



STRATEGY

A) Isolate DNA from a small number of cells
B) Cut with HpaII or HhaI
C) Dilute to single copy (approximately 1 copy per PCR tube)
D) PCR to detect single copy
E) Gel analysis to identify allele based on size
F) Count numbers of each allele
CLONAL= >70% or <30% of each allele
POLYCLONAL= Each allele between 30-70%

ADVANTAGES:

1) Ability to "count" alleles avoids "false" positives due to analysis of too few alleles
2) Allows statistical analysis to identify clonal populations
3) Easily interpreted compared to bulk DNA analysis which requires judgment on whether a given PCR band is stronger than another.

The PCR was optimized using a nesting strategy to amplify single androgen receptor alleles. However, after this optimization, the preliminary results were somewhat disappointing and are summarized below:

	FREQUENCY OF AR ALLELES		
BREAST CANCER	**UPPER**	**LOWER**	**RATIO**
#1: Normal	18	15	55:45
Tumor	54	9	86:14
#2: Normal	23	21	52:48
Tumor	28	11	72:28
MODEL SYSTEMS			
"MONOCLONAL" Hel Cell Line (Breast)	44	14	76:24
"POLYCLONAL" Peripheral White Blood Cells			
Donor #1	16	16	50:50
Donor #2	26	36	42:58

The overall strategy appears sound as normal tissues exhibited the expected proportion of approximately 50% with a range between 58 to 42%. For tumor tissues, the proportion of one allele was, as expected, greater than 50% with a range between 72% to 86%. Therefore, it appears that a proportion of one androgen receptor allele greater than 70% indicates a "clonal" population and a proportion less than 60% indicates a "polyclonal" population.

Of concern was the inability to demonstrate the amplification

of approximately 100% of one allele for the breast cancer cell line (Hel). This general result could not be improved with better restriction enzyme digestion.

Task 2, C,D,E: These tasks have not been accomplished as they were dependent on the success of Task 2B, as noted above.

7) CONCLUSIONS:

Problems have been encountered and we conclude that analysis of small clonal populations by X-chromosome inactivation of the androgen receptor gene is currently unreliable, which may account for the paucity of publications with this potentially powerful approach. Both false positives and negatives occur. The dilution analysis to essentially single androgen receptor alleles shows promise but is extremely time consuming. The exact reasons for the failure to demonstrate absolute "clonality" of the control cell line are unknown but may include such errors as incomplete enzyme digestion prior to PCR, preferential amplification of unmethylated versus methylated DNA, preferential amplification of different sized alleles, and unstable epigenetic methylation patterns in neoplastic cells.

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9) **LIST OF PERSONNEL FROM NEGOTIATED EFFORT**

Darryl Shibata
Quiping Shu
Jenny Tsao

PUBLICATIONS AND ABSTRACTS: NONE